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Monitoring of efficacy of antimicrobial products during 60 days storage simulation of diesel (B0), biodiesel (B100) and blends (B7 and B10)

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HIGHLIGHTS

- The performance of both antimicrobial agents was negatively affected when the aqueous phase was natural bottom water (pH 5).
- **MBO** and **MIT/CMIT** were able to control biomass growth in all fuel grades with synthetic water and low contamination.
- In fuels treated with 1000 ppm of **MIT/CMIT**, the biomass reduction was **lower** than fuels treated with 400 ppm of same product.
- The efficacy of **MBO** in association with other fuel additives, in a multifunctional package was severally reduced.

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ABSTRACT

Microorganisms can cause many operational problems, particularly, during storage and handling of fuel systems. The susceptibility of diesel systems to microbial contamination has been studied for many years but the introduction of biodiesel (Brazil- B5) has raised the incidence of problems in tanks around the world. Among the mitigation alternatives, biocides have been identified as a good one to curb microbial growth. The aim of this research was the effectiveness assessment of two biocides a MBO antimicrobial agent (as multifunctional package) and MIT/CMIT antimicrobial agent in biodiesel (B100 – 60% soya and 40% tallow), conventional diesel (B0 – low sulfur 50 ppm) and blends B7 and B10. The efficacy of two biocides was determined by evaluating the changes in a set of parameters during 60-days exposure to an uncharacterized microbial inoculum. Microcosms contained a fuel phase (B0, B100, B7 or B10) and two types of aqueous phase: natural bottom-water formed in B5 storage tank or a synthetic water with three levels of contamination: low (10^3 CFU L^{-1}), medium (10^5 CFU mL^{-1}) and high (10^8 CFU mL^{-1}). Fuel phase as received and without biocide with sterile aqueous phase was used as a control and the sampling times were at 0, 7, 14, 21, 42 and 60 days. The fuel phase was rated by Haze scale (ASTMD 4176) and infrared analysis. Water phase parameters included: microbial viability (time-kill), presence of emulsion/biofilm and dry weight of biomass formed at fuel/water interface. The results suggest that antimicrobial MBO product as a multifunctional package can only control the microbial population when the microbial contamination level is low. The antimicrobial MIT/CMIT product was effective in all conditions (as received, media and high microbial contamination).

1. Introduction

The use of alternative energy sources has been stimulated worldwide due to concerns with high levels of greenhouse gas emissions, air pollution and dependence on fossil fuels. Biodiesel has been introduced by several countries to contribute to a cleaner atmosphere. In Brazil biodiesel was first introduced into the market in 2004. Since 2010, Brazilian diesel vehicles have been using a B5 blend, with predominantly soya biodiesel. Biodiesel is a good substitute for petroleum and diesel because of its better biodegradability and lower emission of CO_2. However, biodiesel, especially the predominant type in the Brazilian market (soya), is extremely unstable, particularly when exposed to moisture and oxygen from the air [1–3]. Reaction with oxygen leads to the formation of soluble and insoluble oligomers in the form of deposits and sludge, causing clogging and economic losses. Because of the hygroscopic

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nature of biodiesel, water accumulates in fuel that is stored for long periods. High levels of dissolved water lead to the formation of micro-droplets, which culminates in the production of a second phase of free water in the bottom of the tank, a fundamental condition for the proliferation of microorganisms. Historically, uncontrolled microbial contamination during storage has been perceived as a chronic problem in diesel fuel [4–7]. With the introduction of biodiesel into diesel, an increase in the susceptibility to microbial growth has been observed [8–11]. This leads to important consequences: filter clogging, presence of bio-sediments and corrosion influenced by microorganisms [12]. Other consequences, such as reduction in chemical stability or increased corrosivity of the fuel, usually occur together [13]. These degenerative processes are observed by the producers, traders and distributors of fuels, and require doubled analytical attention, continuous monitoring and adoption of mitigation measures, particularly the use of additives such as antioxidants [1]. The hot and humid conditions that prevail in much of Brazil, as well as the exposure to cold and temperate climates predominant in southern Brazil, can introduce significant changes to the original characteristics of the product. If preventive and control actions are not taken, as recommended in Brazilian standard ABNT NBR 15512 [14], a biodiesel originally produced in accordance with the Brazilian specification may become unsuitable for addition to diesel and inappropriate for use as B5 blends [1].

Fuel can be preserved by applying chemicals (biocides) that inhibit and control microbial growth [3,15,16]. Although the details of antimicrobial strategies differ from system to system, one of the key parameters is the choice of antimicrobial product; this should: kill microbes in the fuel and water phase, have a broad spectrum of activity (against fungi, aerobic and anaerobic bacteria), maintain its inhibitory effect in the presence of other substances in the environment in similar operating conditions, not be corrosive to the system, be low cost, be safe to use and have low environmental impact [6,9,10,13]. The requirement to use fuel with the lowest possible sulfur content has resulted in demands for sulfur-free antimicrobial products. Biodegradability and the absence of heavy metals are also required. Some companies guarantee that if the antimicrobial product is properly used, it will be transformed into products of combustion by the engine along with the fuel [9].

The use of antimicrobial products in fuels and biofuels has been recommended in the United States and Europe [6,9,17,18]. Two widely used fuel antimicrobial products currently approved for use by the US military under Military Specifications (MIL SPEC) are based on isothiazoline (MIT/CMIT) and oxazolidine (MBO) chemistries [16]. Both of these have been submitted for approval under the BPD (Biocidal Products Directive – Europe) procedures. However, isothiazolines are not permitted under national German regulations, which restrict use of chlorinated fuel additives [18].

The aim of this study was the evaluation in biodiesel (B100 – 60% soya and 40% beef tallow), conventional petrodiesel S50 (B0 – low sulfur 50 ppm) and B7 and B10 diesel/biodiesel blends of the antimicrobial performance of a multifunctional additive for fuel with antimicrobial agent (MBO) and a conventional antimicrobial product (MIT/CMIT).

2. Materials and methods

2.1. Microcosms

Triplicate microcosms were prepared for destructive testing. The aqueous phase (20 mL) consisted of either *synthetic bottom-water* (g L^{-1}: KCl, 0.7; KH_2PO_4, 2.0; Na_2HPO_4, 3.0; NH_4NO_3, 1.0 plus 1 ml micronutrient solution g L^{-1}: $MgSO_4$, 4.0; $FeSO_4$, 2.0; $MnCl_2$, 0.2; $CaCl_2$, 0.2 – pH 7.2), or *natural bottom water* (pH 5.0) that had been collected from fuel (S500 diesel) storage tanks. This aqueous phase was dispensed into 250 mL flasks with 60 mL fuel. The microcosms were incubated in the dark at room temperature (30 ± 3 °C) for 60 days.

2.2. Fuels

All fuels were provided by Ipiranga Produtos de Petróleo, SA (Brazil). The fuels utilized were: diesel (B0, LSD 50 ppm); biodiesel (B100) from soya and tallow (60:40) and the diesel/biodiesel blends B7 and B10, which were prepared in the lab. All fuels utilized were dispensed into the flasks as received, without sterilization. Fuel was stored in the same room, under the same conditions as the microcosms throughout the study.

2.3. Antimicrobial products (Table 1)

The **MIT/CMIT** antimicrobial product is a well known and studied antimicrobial agent, widely used in fuels and fuel system treatment; it is effective at 100–400 ppm of product. It was used here as a comparative standard and therefore the dosages used were the same as those chosen for **MBO** antimicrobial product (400–1000 ppm). **MBO** has only recently been approved for use in fuels and fuel systems and there are few studies [9,19–21] showing its performance (in diesel fuel only). In this research it was used as part of a multifunctional package associated with other additives for diesel and diesel/biodiesel blends at 400 and 1000 ppm in the fuel phase. These concentrations were determined after a susceptibility test with isolated microorganisms, with an uncharacterized microbial consortium and with the manufacturer's guidance.

2.4. Inoculum

An uncharacterized inoculum was produced as suggested in ASTM E1259-10 [22]. Briefly, an Erlenmeyer flask containing 100 mL of Bushnell–Haas broth [23] supplemented with 2% sterile B10 blend fuel was inoculated with 5 mL of microbial sludge mix obtained from different fuels (diesel, biodiesel and diesel/biodiesel blends B4 and B5) and incubated at 28 °C, 200 rpm for 7 days.

2.4.1. Microbial challenge levels

The synthetic water was evaluated with three microbial challenge levels (low, medium and high) and the natural bottom-water (from storage tanks) with two (low and medium microbial challenge). ***Low microbial challenge*** – fuel as received – 10^3 CFU/L, with sterilized synthetic water or non sterile natural bottom water; ***Medium microbial challenge*** – fuel as received with synthetic water 10^3 CFU/mL fungi and 10^5 CFU/mL bacteria and yeasts; ***High microbial challenge*** – fuel as received with synthetic water 10^6 CFU/mL fungi and 10^8 CFU/mL bacteria and yeasts. In order to obtain the high microbial challenge the inoculum prepared as above was adjusted by spectrophotometry to a 0.5 McFarland turbidity standard (wavelength 530 nm) using Bushnell–Haas broth. This was then diluted 1:1000 to produce the medium challenge (1×10^3 CFU/mL fungi and 5×10^5 CFU/mL bacteria and yeasts) [24–27]. The microbial contamination in fuel "as received" was determined according to IP385 [28]. Samples were taken at 0, 7, 14, 21, 42 and 60 days. Fuel "as received" was utilized as control without inoculum or antimicrobial products.

2.5. Analysis

At each sampling time fuel, water and interface were analyzed separately.

Table 1
Evaluated antimicrobial products.

Biocide	**MBO**	**MIT/CMIT**
Antimicrobial active ingredient	3,3-Methylenebis (5-methyloxazolidine)	5-Chloro-2-methyl-4-isothiazolin-3-one + 2-Methylisothiazol-3(2*H*)-one
Abbrev.	MBO	MIT/CMIT
% Active ingredient (i.a.)	50	1,5
Substance group	N-formal	Isothiazolinones
Manufacturers use concentrations (ppm)	400–1000	100–400
Manufacturers use concentrations (ppm) active ingredient	200–500	1, 5–6
Evaluated concentrations (ppm) product	400–1000	
Evaluated concentrations (ppm) active ingredient	200–500	6–15
Biodegradability	Readily biodegradable (OECD301A, 301D)	Inherently biodegradable. Does not pass readily biodegradable (OECD301A, 301D)
Sulfur content	No sulfur, no halogens, no heavy metals (on MBO)	1, 4 ppm S delivered in 400 ppm
Observations	This product is a blend between a MBO biocide and fuel stabilizers	Product with biocide properties only

2.5.1. Fuel phase

Fuel turbidity was measured using the Haze rating (ASTM D 4176-04) [29]. The chemical changes in the fuel phase were analyzed by FTIR. FTIR spectra were obtained using the Perkin Elmer Spectrum 400 spectrophotometer equipped with a deuterated triglycine sulfate (DTGS) detector and a horizontal attenuated total reflectance (HATR) accessory with zinc selenide crystal (ZnSe). Duplicate spectra were collected from 650 to 4000 cm^{-1} at room temperature. The optical resolution of the IR spectra was 4 cm^{-1} and 16 scans were accumulated for each spectrum. Background spectra in air were obtained for every sample immediately before collecting the sample spectrum. The data were pre-processed by the mean centering method. IR data were subsequently analyzed by Hierarchical Cluster Analysis (HCA) and Principal Components Analysis (PCA) in the spectral region from 1800 to 650 cm^{-1} using MATLAB 7.11 software (The Mathworks) and PLS_Toolbox 6.0 software (Eigenvector Research).

2.5.2. Aqueous phase

Time-kill tests were performed by monitoring viable cells in a 96 well plate with bacterial, fungal and total microorganism growth media. Briefly, a 10 μL aliquot of the water or fuel was removed from each microcosm and added to the wells with 240 μL of culture medium to neutralize the active ingredient. Plate count, malt and nutrient broth were used. The plates were incubated at 28 °C and results read after 2 days for bacteria and 10 days for fungi. The absence of turbidity in the wells was measured as no growth (–). The well with no inoculum constituted the control. The water phase was rated also for turbidity by Haze rating (ASTM D 4176-04) [29], and gross observations (clean or cloudy) were made according to Passman and Dobranic [30].

2.5.3. Interface

The fuel–water interface was rated for the presence of a foam layer, pellicle (membrane) layer or both, as suggested by Passman and Dobranick [30]. Interface characteristics were reported as presence (Y or N) and consistency (flocculent, membranous). At the end of the experiments, after 60 days of incubation, the entire contents of each microcosm (80 mL) were separately filtered through previously weighed filter paper discs. To remove adhered fuel from the biomass, discs were filter–washed with 4 mL of hexane. They were then placed at 30 °C for 48 h and transferred to a dehydrating chamber for 24 h to remove water and the dry weight was recorded. Biomass weight was calculated as final weight minus initial weight (mg). The final weight was obtained by the weight average of triplicates for each treatment.

Table 2
Time (days) for control of microorganisms growth in the water phase **(synthetic water)** with antimicrobial products.

Concentration (ppm)		400		1000	
Fuel phase	Microbial challenge level	**MBO**	**MIT/CMIT**	**MBO**	**MIT/CMIT**
B0	Low	21	7	14	7
	Medium	–[a]	7	21	7
	High	–[a]	7	21	7
B7	Low	21–42	7	21	7
	Medium	–[a]	7	–[a]	7
	High	–[a]	14	–[a]	7
B10	Low	21	7	42	7
	Medium	–[a]	7	–[a]	7
	High	–[a]	7	–[a]	7
B100	Low	21	7	7	7
	Medium	–[a]	7	21	7
	High	–[a]	7	–[a]	7

N° Microbicidal effect; N°-N° inhibitory effect.
[a] Uncontrolled growth, no effect.

Table 3
Time (days) for control of microorganisms growth in the water phase **(natural bottom-water)** with antimicrobial products.

Concentration (ppm)		400		1000	
Fuel-phase	Microbial challenge level	**MBO**	**MIT/CMIT**	**MBO**	**MIT/CMIT**
B0	Low	7–21	7	7	7
	Medium	7–21	21–42	7–21	7
B7	Low	–[a]	21–42	14–42	7–42
	Medium	–[a]	21–42	–[a]	7–42
B10	Low	–[a]	21–42	14–42	21–42
	Medium	–[a]	21–42	14–42	7–42
B100	Low	7–21	7–42	7	7–42
	Medium	7–21	7–42	–[a]	7–42

N° microbicidal effect; N°-N° inhibitory effect.
[a] Uncontrolled growth, no effect.

3. Results and discussion

3.1. Type and time of action and duration of preservation

Biocides are not used to control microbial growth in fuels in Brazil, even though additive packages, containing surfactants, corrosion inhibitors, improvers of cetane number, etc. [31], which claim to improve diesel quality, exist. One of the key parameters is the choice of antimicrobial product, which should have a broad spectrum of activity (against fungi, aerobic and anaerobic bacteria) and be effective against microorganisms in the planktonic and sessile (biofilm) state. Only low molecular weight neutral or cationic biocide molecules may be expected to diffuse, freely from an aqueous solution across the gel formed at the interface and within the gel matrix [5,32]. The biocides used in fuel protection can be classified into two categories: water soluble biocides, and fuel soluble biocides. The fuel soluble biocides, with a partition coefficient (*K*p) between 0.5 and 80, are the most recommended biocides because they allow a better distribution of the active ingredient between the aqueous phase and fuel [11,33,35]. Currently, isothiazolinone (MIT/CMIT) and oxazilidine (MBO) blends are fuel soluble biocides approved for use by the US military under Military Specifications (MIL SPEC) [16]. Despite this, previous studies [20,30,32–42] have shown that many parameters may affect biocidal activity, such as contact time, concentration, fuel/water ratio, inoculum, fuel grade and aqueous phase chemistry. In this case the performance of each antimicrobial product mainly depended on the type of aqueous phase, but fuel grade and microbial challenge level also showed some differences. The results are presented in Tables 2 and 3.

3.1.1. Synthetic bottom water (Table 2)

Three microbial challenge levels were evaluated with this aqueous phase. The synthetic mineral medium contains nutrients such as phosphorus, nitrogen, microelements and has a pH of 7.2. This is

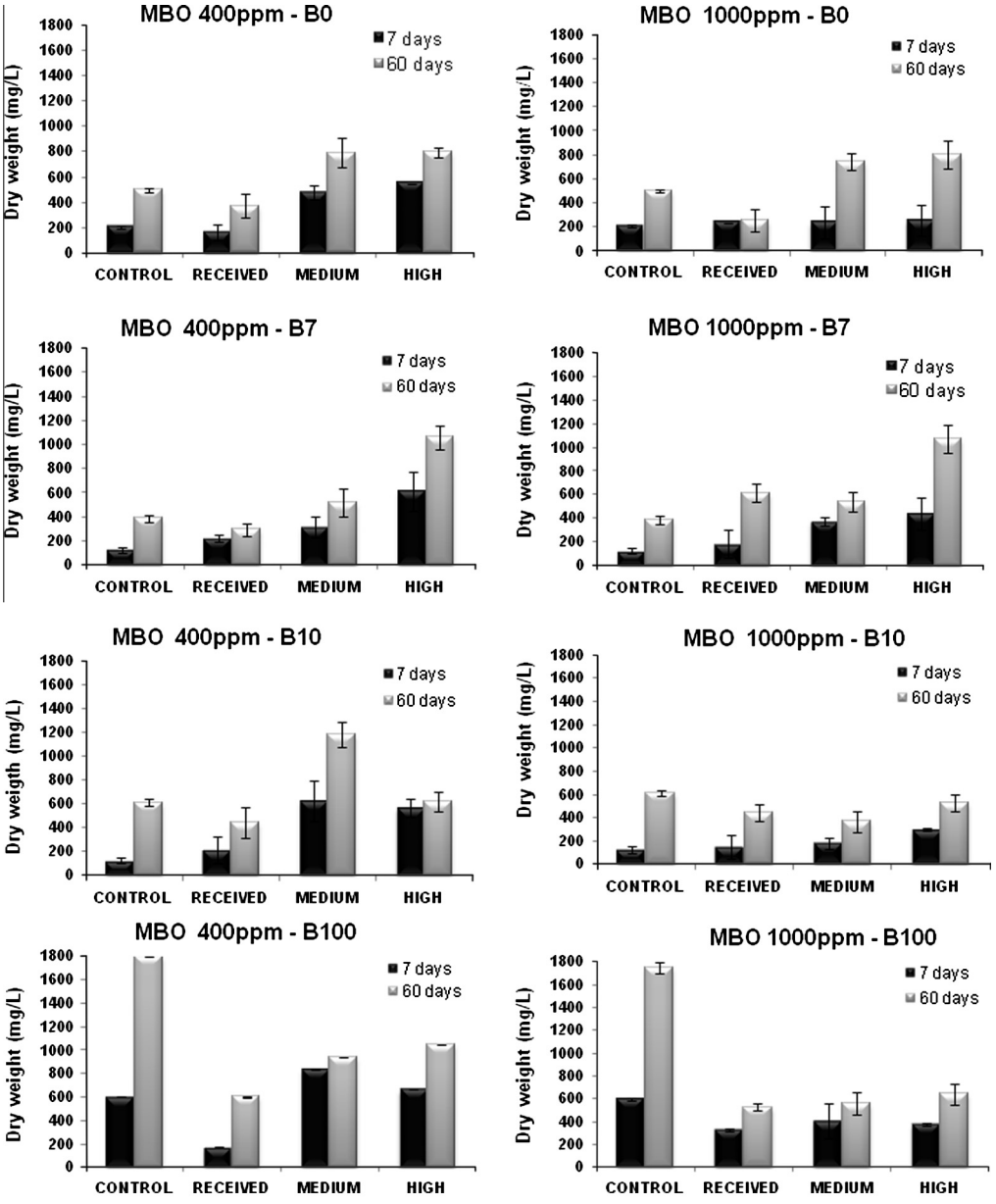

Fig. 1. Biomass formed at 7 and 60 days in B0, B7, B10 and B100 with different levels of microbial challenge (as received, medium and high), with 400 and 1000 ppm antimicrobial **MBO** product.

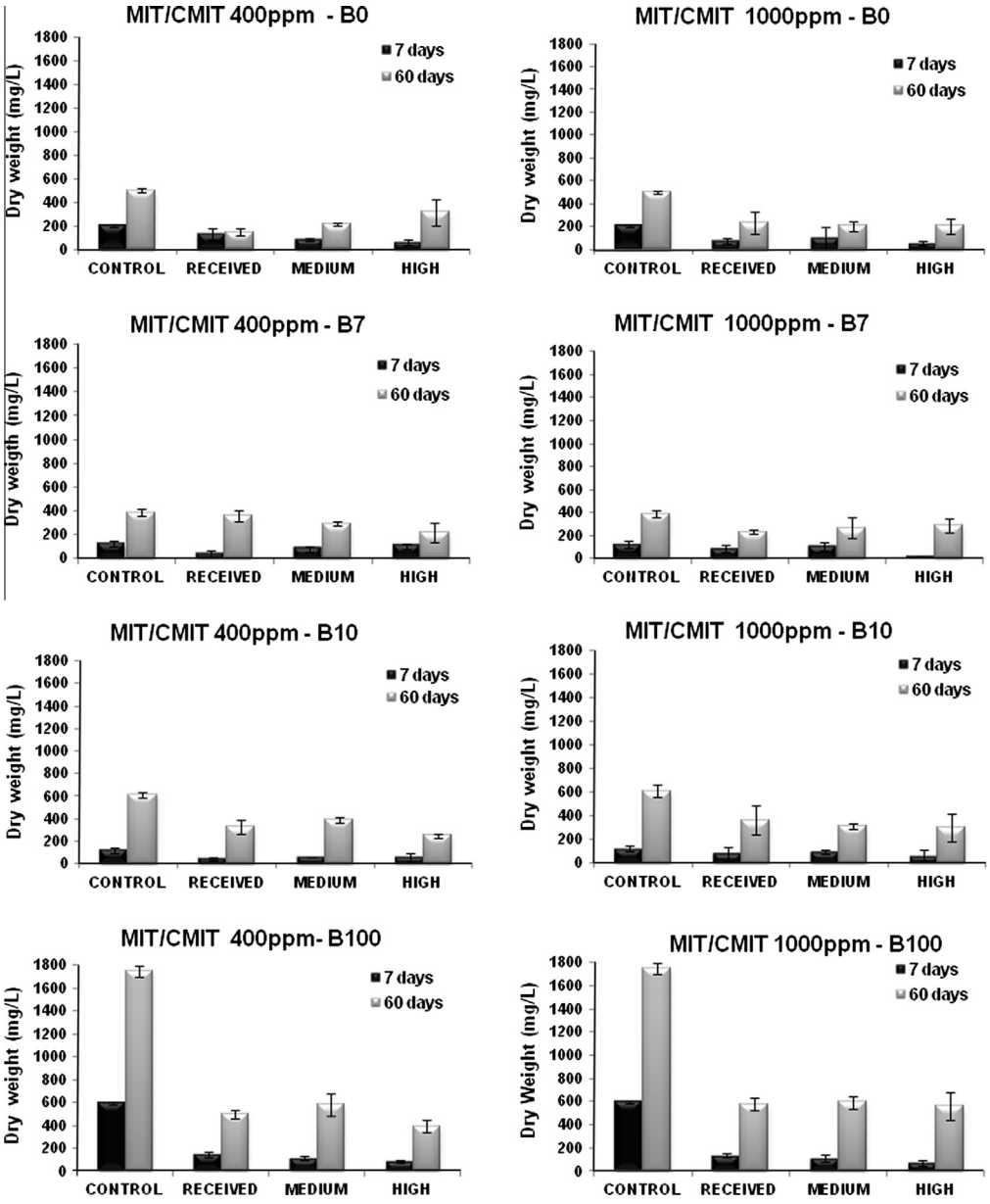

Fig. 2. Biomass formed at 7 and 60 days in B0, B7, B10 and B100 with different levels of microbial challenge (as received, medium and high) with 400 and 1000 ppm antimicrobial **MIT/CMIT** product.

Table 4
Percentage reduction of biomass formed between control and low challenge.

Fuel type	**MBO**		**MIT/CMIT**	
	400 ppm (%)	1000 ppm (%)	400 ppm (%)	1000 ppm (%)
0	26	49	71	53
7	24	–	7	40
10	27	28	46	40
100	66	70	72	67

Table 5
Percentage reduction of biomass formed between control and high challenge.

Fuel type	**MBO**		**MIT/CMIT**	
	400 ppm	1000 ppm	400 ppm (%)	1000 ppm (%)
0	–	–	36	59
7	–	–	43	25
10	–	–	58	51
100	42%	64%	78	68

the aqueous phase most frequently used for antimicrobial evaluation tests for fuel, as it has ideal conditions to support microbial growth. In this fluid, **MIT/CMIT**, at both 400 and 1000 ppm, was quickly effective to control all challenge levels of microbial growth in all fuel grades, as has previously been observed [5,20,30,32,35,39–41].

In the same medium, 400 ppm **MBO** showed a microbicidal effect for the low microbial challenge after 21 days (Table 2) in all fuel grades except for **B7** blend where only an inhibitory effect was observed. No inhibitory effect was observed for medium and high microbial challenge levels for any fuel grade at 400 ppm. At 1000 ppm, a microbicidal effect was observed in **B0** at all challenge levels, in **B**100 for low and medium challenge and in **B7** and **B10** for low microbial challenge. These results disagree with those reported by Passman et al. [20] and Siegert [9] for diesel fuel, where the antimicrobial **MBO** product could control microbial growth at a

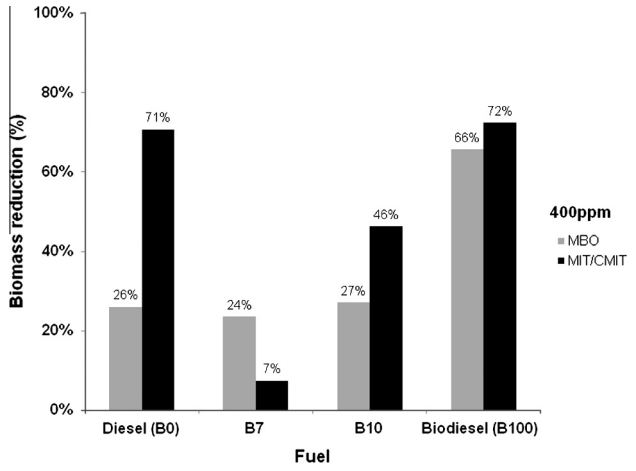

Fig. 3. Percentage reduction of biomass formed in the experimental flasks with aqueous phase consisting of synthetic medium after 60 days between control (no biocide) and fuel treated with 400 ppm of antimicrobial **MBO** and **MIT/CMIT** product.

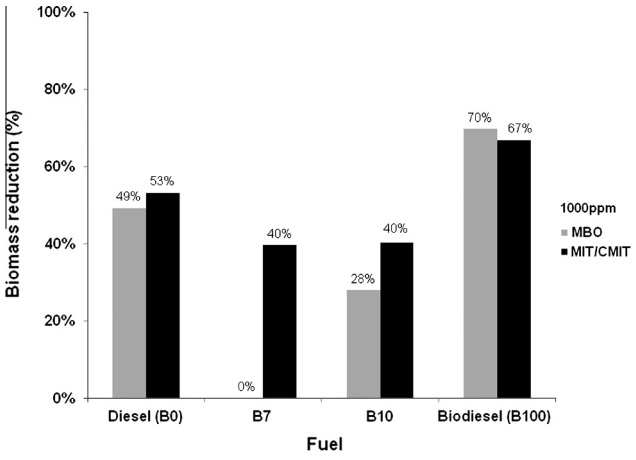

Fig. 4. Percentage reduction of biomass formed in the experimental flasks with aqueous phase consisting of synthetic medium after 60 days between control (no biocide) and fuel treated with 1000 ppm of antimicrobial **MBO** and **MIT/CMIT** product.

microbial challenge level of 10^5 CFU/mL with pure or mixed bacteria, while only 400 ppm was effective against fungi. The possible explanation for these differences is the composition of the **MBO** product, which is not merely a biocide but a multifunctional package, containing other additives that improve fuel and biofuel performance. Geva et al. [21] observed that the presence of an NBM based biocide and a fuel additive in the same product resulted in biocide deactivation. The authors suggested that a possible interaction between additive and biocide in the mixture adversely affected biocide partitioning, preventing the dispersal of active ingredient in the aqueous phase. Similarly, in this study the interaction between additives and MBO probably affected biocide partitioning, but only reduced both the transfer speed of the active ingredient and its availability in the aqueous phase, since the **MBO** product showed antimicrobicidal action at 1000 ppm for all fuel grades after 21 days.

3.1.2. Natural bottom water (Table 3)

Two microbial challenge levels were evaluated in this aqueous phase. The natural bottom water was used to simulate real conditions in a storage tank, as suggested in ASTM 1259-10. This aqueous phase contained mineral elements such as phosphorus, nitrogen, potassium, sulfur and some metals such iron, copper, zinc and manganese and its pH was 5.0, but in real conditions these features can vary widely. Generally, only a transitory inhibitory effect could be observed for the **MBO** and **MIT/CMIT** products in this aqueous phase. The inhibitory effect for **MBO** lasted for 14 and 28 days at 400 and 1000 ppm, respectively, and for **MIT/CMIT** it lasted for 21 and 35 days at 400 and 1000 ppm, respectively (Table 3). The poor performance for both antimicrobial products may be explained by chemical interactions within the aqueous phase at this low pH (5.0) [36]. However, since complete deactivation did not occur, the most probable explanation is that the reduced pH resulted in modified partitioning of the biocides, reducing both the transfer speed of the active ingredient and its availability in the aqueous phase. According to Hill et al. [42], pH is a critical factor affecting biocide performance. **MIT/CMIT** was effective in **B0** fuel at 400 ppm for the low microbial challenge and 1000 ppm for both other microbial challenge levels. At 400 ppm an inhibitory effect only was observed between 7 and 42 days for **B100** and between 21 and 42 days for **B7** and **B10**. For **B0** and **B100**, 400 ppm **MBO** showed inhibitory action up to 21 days for both low and medium microbial challenge levels. At 1000 ppm, a microbicidal effect was observed but only for the low microbial challenge. For blends **B7** and **B10**, no inhibitory effects were observed at 400 ppm. At 1000 ppm inhibition was observed between 14 and 42 days. There was no effect for blends **B7** and **B10** treated with 400 ppm MBO.

Table 6
Microcosms in B0 treated with antimicrobial products (MBO and MIT/CMIT) at 400 and 1000 ppm and control.

B0	Antimicrobial product	MBO				MIT/CMIT			
Challenge level	Concentration	1000 ppm		400 ppm		1000 ppm		400 ppm	
	Time (days)	7	60	7	60	7	60	7	60
Low	Haze (fuel)	3	1	2	1	2	1	3	1
	Interface	N	N	N	N	N	N	N	N
	Bottom water	Clean	Clean	Clean	Cloudy	Clean	Clean	Clean	Clean
Medium	Haze (fuel)	3	1	2	1	2	2	3	1
	Interface	N	N	Floculate	Thick	N	N	N	N
	Bottom water	Clean	Cloudy	Cloudy	Cloudy	Clean	Clean	Clean	Clean
High	Haze (fuel)	2	1	2	2	2	3	2	1
	Interface	N	N	Floculate	Thick	N	N	N	N
	Water bottom	Clean	Cloudy	Cloudy	Cloudy	Clean	Cloudy	Clean	Cloudy
Control-untreated	Haze (fuel)	1	1						
	Interface	N	Foam						
	Bottom water	Clean	Clean						

N: membranous layer not observed.

Table 7
Microcosms in B7. Untreated and treated with antimicrobial products (MBO and MIT/CMIT) 400 and 1000 ppm.

B7	Antimicrobial product	MBO				MIT/CMIT			
Challenge level	Concentration	1000 ppm		400 ppm		1000 ppm		400 ppm	
	Time (days)	7	60	7	60	7	60	7	60
Low	Haze (fuel)	4	2	4	2	2	3	2	1
	Interface	N	Foam	N	Foam	N	N	N	Foam
	Bottom water	Clean	Cloudy	Clean	Cloudy	Clean	Clean	Clean	Cloudy
Medium	Haze (fuel)	6	2	3	2	2	2	1	1
	Interface	N	Thick	N	Thick	N	Foam	N	Foam
	Bottom water	Cloudy	Cloudy	Cloudy	Cloudy	Clean	Cloudy	Clean	Cloudy
High	Haze (fuel)	4	2	6	3	3	2	2	1
	Interface	N	Thick	N	Thick	N	Foam	N	Foam
	Water bottom	Cloudy	Cloudy	Cloudy	Cloudy	Clean	Cloudy	Clean	Cloudy
Control-untreated	Haze (fuel)	2	1						
	Interface	N	Foam						
	Bottom water	Cloudy	Cloudy						

N: membranous layer not observed.

Table 8
Microcosms in B10. Untreated and treated with antimicrobial products (MBO and MIT/CMIT) 400 and 1000 ppm.

B10	Antimicrobial product	MBO				MIT/CMIT			
Challenge level	Concentration	1000 ppm		400 ppm		1000 ppm		400 ppm	
	Time (days)	7	60	7	60	7	60	7	60
Low	Haze (fuel)	3	2	3	1	2	1	2	1
	Interface	N	N	N	Foam	N	N	N	N
	Bottom water	Clean	Cloudy	Clean	Cloudy	Clean	Clean	Clean	Clean
Medium	Haze (fuel)	6	3	2	2	3	1	3	4
	Interface	N	Foam	N	Thick	N	Foam	N	Foam
	Bottom water	Cloudy	Cloudy	Cloudy	Cloudy	Clean	Clean	Clean	Clean
High	Haze (fuel)	4	2	3	3	2	1	2	1
	Interface	Foam	Thick	Foam	Thick	N	Foam	N	Foam
	Water bottom	Cloudy	Cloudy	Cloudy	Cloudy	Clean	Clean	Clean	Cloudy
Control-untreated	Haze (fuel)	4	1						
	Interface	N	Film						
	Bottom water	Cloudy	Cloudy						

N: membranous layer not observed.

Table 9
Microcosms in B100. Untreated and treated with antimicrobial products (MBO and MIT/CMIT) 400 and 1000 ppm.

B100	Antimicrobial product	MBO				MIT/CMIT			
Challenge level	Concentration	1000 ppm		400 ppm		1000 ppm		400 ppm	
	Time (days)	7	60	7	60	7	60	7	60
low	Haze (fuel)	3	3	5	2	2	3	3	1
	Interface	N	N	N	N	N	N	N	N
	Bottom water	Clean	Cloudy	Clean	Cloudy	Clean	Clean	Clean	Clean
Medium	Haze (fuel)	4	2	2	1	1	1	3	1
	Interface	N	N	Thick	Thick	N	N	N	N
	Bottom water	Cloudy	Cloudy	Cloudy	Cloudy	Clean	Clean	Clean	Clean
High	Haze (fuel)	3	2	5	2	1	1	2	1
	Interface	N	N	Film	Thick	N	Thin	N	N
	Water bottom	Clean	Cloudy	Cloudy	Cloudy	Clean	Cloudy	Clean	Clean
Control- Untreated	Haze (fuel)	3	1						
	Interface	N	Thick						
	Bottom water	Cloudy	Cloudy						

N: membranous layer not observed.

The microbicidal effect was observed only for pure diesel fuel (**B0**) at low microbial challenge level for both antimicrobial products, except for **MBO** at 400 ppm. An interaction between pure (**B100**) and blended biodiesel (**B7** and **B10**) could explain the poor performance shown by **MBO** in these fuel grades in both aqueous phases evaluated, but there was not sufficient data to clarify this issue.

3.2. Effect of antimicrobial product on microbial growth

Biomass was measured as dry weight after 7 and 60 days of incubation. All biomass formed in the microcosm was measured at each sampling time (data not shown) and the criterion used to determine preservative potential was the ability of antimicrobial

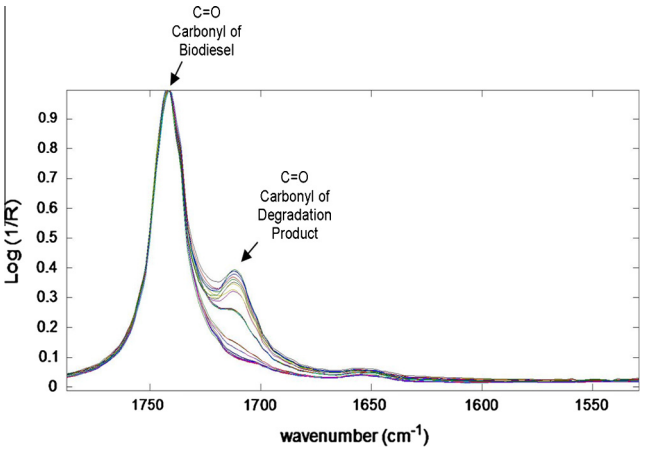

Fig. 5. HATR-FTIR spectra for blend B10 (400 ppm antimicrobial **MBO** product in synthetic mineral medium with high challenge microbial level –108 CFU/mL).

products to lead to reduced biomass after 60 days. For natural bottom water this procedure was not performed. Figs. 1 and 2 summarize the results in B0, B100, and blends B7 and B10 treated with 0, 400 and 1000 ppm **MBO** and **MIT/CMIT** products in synthetic bottom water.

MBO reduced growth when the initial microbial population was low (Table 4); however, with higher microbial densities no antimicrobial action was detected at 400 ppm or 1000 ppm, except in B100 (Table 5). No significant differences were found (*t*-test $p < 0.05$) in the reduction of biomass (%) by fuels treated with 400 or 1000 ppm, except for the pure diesel (B0) and B7 blend, suggesting that 400 ppm would be adequate for a preventive treatment in B100 and blends and B10 when synthetic bottom water is the aqueous phase (Table 4).

MIT/CMIT controlled the biomass at 400 and 1000 ppm at all three levels of microbial challenge. However, the percentage biomass reduction for fuels treated with 1000 ppm **MIT/CMIT** was **lower** than that treated with only 400 ppm (Tables 4 and 5). Raikos et al. [39], working with an **MIT/CMIT** based biocide, also observed that when higher concentrations of biocide were used its effectiveness was reduced. The authors related this fact to changes observed in the partitioning characteristics of the product that resulted in a lower availability of active ingredient in the aqueous phase. According to El-Zanfaly et al. [43], higher concentrations of biocides could require some pH adjustment in order to ensure the activity profile.

The biomass formed in B100 (control) after 60 days was on average 3.5 times higher than that formed in blends B7 and B10 and in pure diesel (B0) (Figs. 1 and 2). Despite this, in the flasks treated with antimicrobial products the biomass increase was greater the higher the percentage of biodiesel in the blends. This suggests that high biomass in the control B100 is more related to the higher susceptibility of biodiesel to microbial degradation than to differences in the performance of the antimicrobials. Biodiesel has been proven to be a much better carbon source for supporting microbial growth than pure diesel [11,44–46].

A significant reduction (*t*-test $p < 0.05$) in biomass growth after 60 days was observed for the low microbial challenge in all fuel grades treated with both antimicrobial products compared to the untreated control (Table 4). In contrast, only **MIT/CMIT** gave reduced biomass after 60 days at high microbial challenge levels (Table 5). Figs. 3 and 4 show the performance of MBO and MIT/CMIT at 400 and 1000 ppm for the low challenge level.

3.3. General observation

The visual analysis of fuel treated with antimicrobial agents is important in selection of the appropriate product. The treatment must not induce fuel turbidity, color changes, or formation of solid particles. Visual analysis can also provide important data on appearance, quantity and distribution of microbial biomass in the microcosms studied, allowing a better understanding of the problem as a whole.

Tables 6–9 summarize the observations made for microcosms B0, B7, B10 and B100. At 60 days, when antimicrobial products controlled the microbial population, the fuel lost some of its color and the Haze value diminished. The appearance of the fuel–water interface varied according to the level of microbial contamination from a well defined, continuous membranous layer to a flocculent and discontinuous zone.

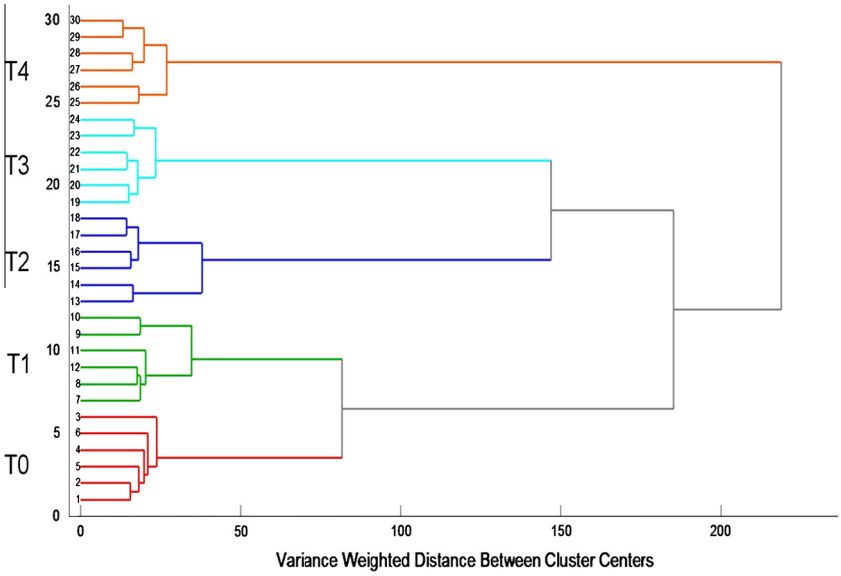

Fig. 6. Dendrogram of the B10 spectra (T0: zero days – red, T1: 7 days – green, T2: 14 days – blue, T3: 28 days – light blue and T4: 60 days – orange). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

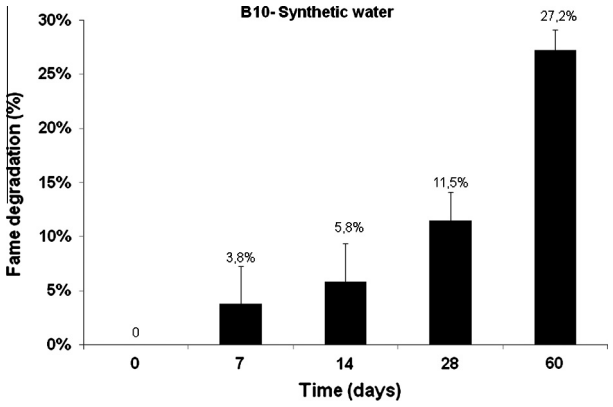

Fig. 7. Percentage degradation of B10 blend treated at 400 ppm of antimicrobial MBO product in synthetic mineral medium after 60 days.

3.4. HATR–FTIR spectra

HATR–FTIR spectra of the fuel phase, analyzed using multivariate techniques (PCA and HCA), showed the degradation suffered by biodiesel when the microbial growth was not controlled. Fig. 5 shows the B10 blend spectra for 400 ppm **MBO** in synthetic water and high challenge microbial level (10^8 CFU/mL), where the microbial growth was not controlled. The signal of the carbonyl group of the biodiesel (1745 cm^{-1}) decreases with time, and a new signal, the degradation product of biodiesel, appears and increases. The dendrogram for these B10 spectra (Fig. 6) shows that the biodiesel is being consumed over time, since the carbonyl band of biodiesel decreases from zero to 7, 14, 28 and 60 days. After 60 days, almost 30% of the biodiesel had been degraded (Fig. 7). For the same treatment, when the microbial growth was controlled by MIT/CMIT, no degradation of the biodiesel was seen after 7 days. These data support the need to use products that promote a rapid and effective control of microbial growth in stored fuels, especially biodiesel and their blends, in order to prevent economic losses.

4. Conclusions

In this study the performance of **MBO** and **MIT/CMIT** antimicrobial products was assessed in a laboratory screening test based on ASTM standard E 1259. The efficacy of both antimicrobial products for diesel (B0), biodiesel (B100) and the blends B7 and B10 was assessed against an uncharacterized inoculum at three different microbial challenge levels and in two different aqueous phases. Our conclusions are mainly based on four aspects: type of action (microbicidal or microbiostatic), time of action, duration of preservation and effect on microbial growth assessed as biomass. From a microbiological point of view only, **MIT/CMIT** showed the best performance. However it is necessary to clarify some points. The performance of both antimicrobial products was adversely affected when the aqueous phase was **natural bottom water**, emphasizing that the system must be drained before treatment with any antimicrobial agent. Both antimicrobial products were able to protect all grades of fuel evaluated, but some interaction between blends B7 and B10 and **MBO**, mainly in **natural bottom water**, might have occurred; this requires further investigation. Previous studies show that **MBO** used alone has a performance comparable to **MIT/CMIT** [5,16–18]. The MBO product used in our study contained many other fuel additives, intended as broad action control for diesel and biodiesel fuels. This appeared to reduce the antimicrobial activity of **MBO**. However, in the experimental conditions evaluated, in synthetic mineral medium and at 400 ppm, it could be considered a good strategy for preventive treatment in diesel and biodiesel fuels.

It is important to consider the problems in fuel as not limited to microbial contamination, but also including the oxidation and loss of lubricity that may cause damage to engines; this reinforces the need for a multifunctional package. The increasing demand for fuel and the introduction of biodiesel will lead to increasing biodeterioration problems. Relatively high levels of biodiesel in fossil diesel are already mandatory in Brazil (B5) and Europe (B7) and even higher percentages are under discussion. This scenario needs to be investigated and taken into account during the engineering of fuel system solutions for the future. Although it was not within the scope of this work, environmental demands are another aspect that has become increasingly relevant and must be seriously examined when choosing a preservative for use in fuels. In this respect, the **MBO** antimicrobial is more environmentally friendly than **MIT/CMIT**, since it is rapidly degraded in the environment and contains no sulfur (Table 1). As regards occupational health, there are no reports of problems involved in the use of **MBO**, unlike **MIT/CMIT**.

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